CHAPTER - II

MATERIAL AND METHODS

A) MATERIAL

The seeds of two promising cultivars of groundnut, W - 44 and SB - 11 were selected. Seeds were surface sterilized steralized with 5% sodium hypochlorite solution for 5 mins, then washed thoroughly with steralized glass distilled water. The seeds were transferred for field cultivation. The field cultivation was conducted during dry (Feb., 2007, sown) cropping seasons. The thirty- days old seedlings were sprayed with different concentrations (5, 50, 100 and 200 ppm) of SA in three - doses by keeping 4 - days interval. The fresh leaf samples were collected on 7th day after the last spray of SA and washed thoroughly with glass distilled water and used for further analysis.

1. Groundnut Cultivar, W-44:

This variety is a western Research Groundnut Germplasm released by Western Agri. Seeds, Ltd., Gandhinagar (Gujarath). By habit variety is semi-spreading type has pale green foliage. The height of plant is about 30 cm. Pod sizes are medium. Kernel is 1-2 seeded and pink in colour. Oil percentage is 48-50%. Duration for maturity is 100 to 105 days. Variety is cultivated in both Kharif and summer season. In Kharif season yield is about 900 to 1000 K/ha while in summer yield is about 1000 to 1100 K/ha in country whereas, in Maharashtra yield of Kharif W-44 is about 1000-1100 K/ha and in summer season is 1400-1500 K/ha.

2. Groundnut Cultivar, SB-11:

This variety is released in 1955 in Maharashtra. It was developed by interverietal cross between selection AH 4218(B) × AH 4354. By habit this variety is bunch type. The height of this variety is 35 cm. Branching habit is generally n+1. Leaflet colour is green and size is larger, pod sizes are small but with a small beak developed at the tip and reticulation on the pod are no so prominent. Kernel is 1-2 seeded. Kernels are small, pink in colour. Oil percentage is about 50%. However duration is 55-60 days. Days for maturity are 105 to 110. This variety is cultivated in both kharip and summer season. Yield is about 1200-1400K/ha.

Plate No. 1. Seeds of Cv. W-44

Plate No. 2. Seeds of Cv. SB-11

B) METHODS

1. GROWTH STUDIES

The influence of foliar application of salicylic acid on growth characteristics of groundnut cultivar, W-44 and SB-11 is studied (Plate No. 3-8). The parameters include hieght, root-shoot lenght and total number of leaves, leaf area per plant, number of branches, number of root nodules, fresh weight and dry weight of root and shoot and number of pods per plant.

2. PHOTOSYNTHETIC PIGMENTS

a) Chlorophylls

The chlorophylls were estimated by the method of Arnon (1949). 1 g of fresh leaves from investigated seedlings was extracted in 80 % acetone. The extract was filtered through Bachner's funnel using Whatman filter paper No-1. Residue was washed repeatedly with 80% acetone. The volume of the filtrate was made to 100 ml by using 80% acetone. Extraction was carried out in dark and in cold conditions. The absorbance of the filtrate was read at 663 nm and 645 nm. Chlorophylls were calculated by using following formula.

Chlorophyll 'a' = $12.7 \times X\ A_{663} - 2.69 \times A_{645} =$ 'X'

Chlorophyll 'b' = $22.9 \times A_{645}\ _\ 4.68 \times A_{663} =$ 'Y'

Total chlorophyll = $8.02 \times A\ 663 + 20.20 \times A_{645} =$ 'Z'

Chlorophyll 'a' or 'b'
Or
Total Chlorophylls (mg g^{-1} Fr.tissue) = $$\frac{X / Y / Z \times \textit{Volume of extract} \times 100}{1000 \times \textit{Wt.of plant material in g}}$$

The values were expressed in mg.100^{-}g fresh tissue.

b) Carotenoids:

Carotenoids were estimated by reading the absorbance of the leaf extract at 480 nm. Total carotenoids were estimated by using the formula given by Kirk and Allen (1965).

$$\text{Carotenoids} = \frac{A_{480} \times \textit{Volume of extract} \times 10}{2500 \times \textit{Wt.of Plant Material}} \times 100$$

Where, 2500 = Average extinction

3. ENZYMES

All enzymes except dehydrogenase and nitrate reductase were studied by using *in vitro* technique. The leaves of all plants from the various SA treatments along with control were selected for the extraction of the enzymes. The extraction and assay of enzymes were performed in the following manner.

a) Enzyme Nitrate Reductase: (E.C. 1.6.6.1)

Activity of Nitrate reductase in groundnut leaves was determined by following the *in vivo* method described by Jaworski (1971). The leaf samples of groundnut seedlings under investigation were taken washed, dried and they were cut into small pieces. 500 mg of leaf pieces were suspended in 10 ml incubation medium containing 1 ml 1 M KNO_3, 2 ml n-propanol (5%), 5 ml 0.2 M phosphate buffer (pH = 7.5), 0.5 % Triton X – 100. The test tubes were sealed and incubated at 25^0C in the dark for 2 hrs. After 2 hrs., Nitrate reductase activity was measured by determining NO_2 production, which was traced by treating 1 ml incubation mixture with 1 ml 1% sulphanylamide (Prepared in 1 N HCL) and NEEDA (N-1 Naphthyl - ethylene diamide Dihydrochloride) for 20 min. The absorbance was measured at 540 nm on spectrophotometer.

The enzyme activity is expressed as μmoles NO_2^- released hr^-1 g^-1 fresh tissue.

i) Glutamate Dehydrogenase (E.C. 1.4.1.2)

The enzyme was assayed by the method of Chou and Splittsloesser (1972).

1g of mature leaves were taken from SA treated groundnut cultivars and crushed in 10 ml of 0.1 M Tris-HCl buffer with pH 8.0. PEG 4000 (0.25% W/V) was added to the crushing buffer to prevent interference by polyphenols. EDTA (1mM), $CaCl_2$ (1 mM) and 2 mercaptoethanol (1 mM) were also added to the crushing buffer, to ensure enzyme stability during the assay. The leaves were homogenized in a prechilled mortar and pestle. Then homogenate were filtered through two layers of musclin cloth. The homogenate was then centrifuged in a refrigerated centrifuge at 5,000 x 9 for 20 min at 0^oc. The supernatant was used as the enzyme source.

Assay:

A total 3ml of assay mixture contained,

0.1 M	Tris HCl pH-8
0.66 mM	2 Oxo - Glutarate
66 μm	NADH
0.1 ml	Enzyme
8.33 mM	NH_4Cl.

The reaction was initiated by the addition of NH_4Cl. The enzyme activity was measured at 340 nm, using MSU spectroplus D. The amount of NADH oxidized was calculated from the standard curve of NADH. Soluble protein content of the enzyme was estimated by the method of Lowry *et al.* (1951). The activity of glutamate dehydrogenase was expressed as μm NADH oxidized/mg protein/min.

c) Oxidative Enzymes

i) Enzyme Peroxidase (E.C 1.11.1.7)

(Donor: H_2O_2 Oxidoreductase)

The activity of peroxidase from fresh plant leaves was determined following the method of Kondo and Morita (1951) as described by Horigunchi (1988). The leaf samples were collected from salicylic acid sprayed control set plants and homogenized in 10 ml of (1/15 M) phosphate buffer (PH 6.8) and filtered through 4 layers of muslin cloth. The filtrate was centrifuged at 10,000 rpm for 20 minutes at 4°C. Supernatant was used as a source of enzyme.

Assay mixture containing 5 ml of 1/15 M Acetate buffer (pH = 5), 0.5 ml of 0.1% Guiaicol, 1 ml enzyme extract, 2ml distilled water, 0.5 ml of 0.08% H_2O_2 were incubated at 30^0c. After 15 minutes of incubation, 1ml of 1N H_2SO_4 was added to terminate the reaction. Reaction mixture was also taken in another test tube and the reaction was immediately terminated by addition of 1 N H_2SO_4. The difference in absorbance at 470 nm of '0' minutes and 15 minutes reaction mixture was measured by using shimatzu double beam spectrophotometer.

The units of the enzymes calculated by the method described by Bergmeyer (1974). The enzyme activity is expressed as min^{-1} g^{-1} protein.

ii) Enzyme Catalase: (E.C. 1.11.1.6)

(Hydrogen peroxide: Hydrogen peroxide oxidoreductase)

1 g of leaf sample from all the treated groundnut plants of both the cultivars viz., W-44 and SB-11 were homogenized in 10 ml of 0.067 M phosphate buffer (pH 6.8). Homogenate were filtered thought 4 layers of muscling cloth. The filtrate was centrifuged at 10,000 rpm for 20 minutes at 4^0C and supernatant was used as a source of enzyme.

The assay of enzyme catalase was carried out following the method of Luck (1974) as described by Sadasivam and Manikam (1992).

Assay mixture contained 3 ml of 0.05 % H_2O_2 [0.16 ml of H_2o_2 (10 % W/V)] were diluted to 100 ml with phosphate buffer (PH = 7) and 0.01 ml of enzyme. The decrease in absorbance during 60 seconds was recorded at 240 nm on schimatzu double beam UV-Visible spectrophometer. The units calculated by the method described by Bergmeyer (1974).

Enzyme catalase activity expressed as Units min^{-1} mg^{-1} protein.

4. STOMATAL CONDUCTANCE AND WATER RELATIONS

1. Stomatal Transpiration

The stomatal transpiration was studied from lower surface of leaves in plants treated with various SA concentrations by cobalt chloride paper method (Because of unavailability of Porometer). The change in colour from blue to pink is a criterion to determine the rate of transpiration. Thus the rate of transpiration was determined by observing the intensity of pink colour of cobalt chloride paper.

2. Moisture Perecentage

Moisture percentage in various plant parts (leaves, shoot and roots) from the SA treated groundnut cultivars, W-44 and SB-11 as well as control plants was determined using following formula,

$$\text{Moisture Percentage} = \frac{Fresh\,Weight - Dry\,Weight}{Fresh\,Weight} X\,100$$

(Weight taken after drying the plant material in oven at 80°C till a constant weight was obtained)

5. INORGANIC CONSTITUENTS

The leaf samples of random sampling were taken, washed thoroughly with glass distilled water and dried in oven at 60°c for about 7- days till a constant weight is obtained. The dried leaf material was powdered.

a) Preparation of Acid Digest

The plant material was digested following the method of Toth *et al.* (1948). An acid digest from the oven dried plant material was used for the estimation of inorganic constituents.

500 mg of oven dried powdered material was transferred to 100 ml beaker, to which 20 ml concentrated HNO_3 were added. The beaker was covered with watch glass and was kept till the primary reactions subsided. It was then heated at low temperature till the solid particles are completely dissolved. After cooling to room temperature 10 ml of perchloric acid (60 %) were added to it and mixed thoroughly.

It was then heated strongly until a clear and colorless solution was obtained. Heating was stopped when the volume of extract was reduced to approximately 2-3 ml. It was then cooled and transferred quantitatively to 100 ml volumetric flask, then diluted to 100 ml with distilled water. It was kept overnight, and filtered through a dry Whatman No. 1 filter paper next day. The filtrate was used as the source of different inorganic constituents.

i) Estimation of Phosphorus:

Phosphorus was estimated calorimetrically following the method of Sekine *et.al.* (1965). Phosphorus can be estimated calorimetrically by comparing the colour intensity of sample.

In test tube 1 ml acid digest, 2 ml 2 N HNO_3 and 1 ml of molybdate vandate reagent were taken. (A: 1.25 g of ammonium molybdate were dissolved in 500 ml 1N HNO_3 , B : 25 g of ammonium vandate were dissolved in 500 ml distilled water. Then A and B were dissolved in equal volumes). The final volume of reaction mixture made to 10 ml by adding distilled water. After shaken well reaction mixture was kept for 20 minutes for full colour development. The absorbance of blank reaction mixture containing no phosphorus was read at 420 nm.

By using various concentrations of phosphorus (0.025, 0.05, 0.1, 0.2, 0.4mg) and standard KH_2PO_4 solution containing 0.025mg phosphorus per ml. the standard

curve of phosphorus prepared. The amount of phosphorus in the plant material was calculated using the standard curve.

ii) Estimation of Potassium and Calcium :

Potassium (K^+) and Calcium (Ca^{++}) concentrations were determined with a flame photometer. Stock solutions of known concentrations in parts per million (ppm) of 'K' in KCl (10 to 50 ppm), Ca in $CaCl_2$ (10 to 200 ppm) and (1 to 10ppm) were used for calibration curves. The concentrations of K, Ca and in acid digested samples were calculated using respective calibration curves.

iii) Estimation of Magnesium, Iron, Manganese and Copper :

Magnesium (Mg^{++}), Iron (Fe^{++}) Copper (Cu^{++}) and Manganese (Mn^{++}) was analyzed by atomic absorption spectrometer (Perkin-Elmer model-3030) using acetylene air flame. The light source employed was hollow cathode lamp. The concentration of Mg^{2+}, Fe^{3+}, Mn^{2+} and Cu^{2+} were react at 285.2 nm, 248.3 nm, 279.5 nm and 324.8 nm respectively.

iv) Estimation of Zinc and Molybdenum :

Zinc was determined by autoanalyser. Molybdenum was estimated by using atomic spectrphotometric method at 313.3 nm.

The values of all macro elements and microelements are expressed in g 100^{-1} g dry tissue.

6) ORGANIC CONSTITUENTS

a) Starch

Carbohydrates were estimated according to the method of Nelson (1944). Fresh leaves (0.3 g) were extracted with 80% ethanol. Then extract was filtered through Buckner's funnel by using Whatman filter paper No. 1. The filtrate was collected and used for the estimation of sugars while the residue was used for the estimation of starch. The filtrate thus collected was condensed to 3-5 ml on water bath in porcelain dish. Then decolorizing agent lead acetate and potassium oxalate (1g : 1g) were added and thoroughly mixed with condensed filtrate. Then about 15 ml of distilled water was added to it and filtered through filtrate paper (Whatman No. 1). The residue was washed for 3 times with distilled water. The final volume of aliquot was measured and

15311

noted down. 20 ml of this extract was hydrolyzed with 2 ml conc. HCl in 150 ml conical flask with a bored cork by autoclaving at 15 lbs pressure for half an hour. The contents were collected, neutralized with sodium carbonate and filtered.

The insoluble residue of starch along with filter paper was transferred to 150 ml conical flask. Then 50 ml distilled water and 5 ml conc. HCl were added. The conical flask was corked with bored cork with bored cork and contents were hydrolyzed by autoclaving at 15 lbs. pressure for half an hour. The contents were cooled, neutralized with sodium carbonate and filtered through Whatman filter paper No. 1. The final volume was measured and noted down. This filtrate contains reducing sugars produced as a result of hydrolysis of starch. These sugars were estimated.

The appropriate quantity of (0.5 ml) of extract in 10 ml marked test tubes. In other such test tubes different concentrations (0.1ml, 0.2ml, 0.3ml,0.4ml and 0.5ml) of std. glucose solutions were taken (standard glucose solutions contains 0.1mg glucose ml^{-1} distilled water). 1ml of somogyi's alkaline copper tartarate reagent (4 g of $Cuso_4$, $5H_2O$, 24 g of anhydrous Na_2Co_3, 16 g of Na-K-tartarate) (Rochella salt) and 180 g of anhydrous Na_2SO_4 dissolved in 1 lit. of D.W. was added to each test tube. All test tubes containing mixtures were kept in water bath for about 10 minutes. Afterwards they were cooled to room temperature and 1 ml of arsenomolybdate reagent (25 g of ammonium molybdate $\{(NH_4)_6\ Mo_7\ O_{24}, 4H_2O\}$ was dissolved in 450 ml of D.W. To it was added 21 ml of conc. H_2So_4 to this solution, then, was added 3 g of sodium acetate ($Na_2H_2SO_4$, $7H_2O$) dissolved in 25 ml of H_2O. All ingredients of reagent were mixed well and reagent was kept in incubator at 37°C for 24 hours before use. The reagent was stored in brown bottles was added to each lest tube with the help of burette. The contents of each test were then diluted to 10 ml with D.W. The blank was prepared by the same way but without any sugar solution. After 10 min. absorbance of each reaction mixtures was read at 560 nm on (double beam spectrophotometer UV-190) Shimadzu. The standard curve was used for calculating the amount of sugars in various samples. The values are expressed as g100 g^{-1} dry tissue.

b) Total Nitrogen and Crude Prodeins

Total nitrogen from the leaves of groundnut subjected to different concentrations of SA treatments was estimated according to method of Hawk et al. (1984). Oven dried 1g powdered plant material was taken in Kjeldahl flask with a pinch of micro salt (200g K_2SO_4 + 5g $CuSO_4$, dehydrated) and to it 5 ml of H_2SO_4 (1:1) were

added. Few glass beads were added to avoid bumping and material was digested on blue flame till it become yellow in colour. The flask was then cooled to room temperature. 15 to 20ml distilled water were added and with thorough shaking the content was filtered and transferred to volumetric flask and volume of filtrate was made 100ml with distilled water.

In clean and dry Nessler's tube 1 ml of plant extract and different concentrations of standard ammonium sulphate solution (0.236 g of ammonium sulphate dissolved in water and few drops of $H_2 SO_4$ were added. The volume is made 1000 ml. This solution contains 0.05 mg of nitrogen per ml.) In blank nessler's tube 1 ml distilled water was taken. To this drop of 8% KHSO4 was added and volume was made 35 ml with distilled water. To this 15 ml Nessler's reagent were added. (Reagent A: 7g KI + 10 g HgI_2 in 40 ml distilled water. Reagent B: 10g NaOH in 50 ml distilled water. A and B were mixed in proportion of 4:5 at the time of estimation) the reaction between the sample and the reagent gives the product $NH_4Hg_2I_3$ which has orange brown colour. This colour was measured after 15 min at 520 nm on double beam spectro photometer (Shimadzu UV 190). Amount of nitrogen was calculated from the standard curve.

Protein content was calculated by multiplying the total nitrogen content by the factor 5.46, specified for nitrogen content in groundnut by Sadasivam and Manikam (1992).